PATENT SPECIFICATION

732,557

Date of Application and filing Complete Specification: July 21, 1952
No. 18415/52.
Application made in United States of America on July 27, 1951.
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Index at acceptance:—Class 2(3), U4(A2:C5:X), V.

COMPLETE SPECIFICATION

Microbiological Oxidation of Steroids

SPECIFICATION NO. 732,557

By a direction given under Section 17(1) of the Patents Act 1949 this application proceeded in the name of Olin Mathieson Chemical Corporation, a corporation organised and existing under the laws of the State of Virginia, United States of America, of 745, Fifth Avenue, New York 22, State of New York, United States of America.

THE PATENT OFFICE,
10th February, 1956

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been made to convert steroids into medicinally-useful substances by utilizing the activity of growing microorganisms, but with generally unsatisfactory results from the standpoints of utility of the substance produced and/or efficiency of its production. Thus, the prior attempts resulted either in oxidation of hydroxy groups to keto groups (as in the conversion of dehydroepiandrosterone to androstenedione) or rupture of the steroid nucleus, rather than the desired addition of oxygen to the steroid nucleus.

It has now been found that steroids, especially 3-keto-steroids, can be converted into useful derivatives by subjecting them to the action of enzymes of a special microorganism under aerobic conditions. This microorganism is an actinomycete isolated from a soil sample from Yonkers, New York. When grown on synthetic or natural media, it produces an antibiotic inhibiting gram-positive and gram-negative bacteria; and when grown in media supplemented with as the first oxidation product.

The microorganism, when grown on agar, has mature vegetative hyphae whose diameter varies from 0.9 to 1.2 microns. The aerial mycelium is hyaline under the microscope, generally branched, not forming loops or spirals. Individual filaments are rarely or not septate. The colour of colonies when viewed on agar without magnification is white to light gull grey (Ridgway plate I. III 10 f). The spores are oval to oblong. Mature spores range from about 1.0 to 1.2 microns in diameter and from 1.0 to 1.2 microns in length. Individual spores are colorless at maturity, but in mass appear white to grey when viewed without magnification (Ridgway plate L III 10 f).

The microorganism will liquefy gelatine, peptonize litmus milk, reduce nitrate to nitrite, and produce hydrogen sulphide when grown on Kligler iron agar. It does not produce indole when grown on tryptone agar. It will grow on

[*Price 3s. 0d.*]

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COMPLETE SPECIFICATION

Microbiological Oxidation of Steroids

We, E. R. SQUIBB & SONS, a corporation organized and existing under the laws of the State of New York, United States of America, of 745 Fifth Avenue, New York, 22, New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to steroids and derivatives thereof, and has amongst its objects the provision of novel derivatives of steroids and methods of producing the same. The invention is primarily concerned with the production of medicinal agents (or intermediates for medicinal agents) from steroids by microbiological action.

Prior to this invention, attempts had been made to convert steroids into medicinally-useful substances by utilizing the activity of growing microorganisms, but with generally unsatisfactory results from the standpoints of utility of the substance produced and/or efficiency of its production. Thus, the prior attempts resulted either in oxidation of hydroxy groups to keto groups (as in the conversion of dehydroepiandrosterone to androstenedione) or rupture of the steroid nucleus, rather than the desired addition of oxygen to the steroid nucleus.

It has now been found that steroids, especially 3-keto-steroids, can be converted into useful derivatives by subjecting them to the action of enzymes of a special microorganism under aerobic conditions. This microorganism is an actinomycete isolated from a soil sample from Yonkers, New York. When grown on synthetic or natural media, it produces an antibiotic inhibiting gram-positive and gram-negative bacteria; and when grown in media supplemented with cobalt salts, relatively large quantities of vitamin B_{12} are formed. These properties of the microorganism, though utilizable in the methods of this invention to obtain the antibiotic and/or a vitamin B_{12} as a by-product, are of secondary importance, the invention being based primarily on the ability of enzymes of the microorganism to produce useful derivatives of steroids subjected to their action under aerobic conditions.

The action of the enzymes can be utilized either by including the steroid in an aerobic culture of the microorganism, or by bringing together, in an aqueous medium, the steroid, air, and enzymes of non-propagating cells of the microorganism. Thus, when supplemented with progesterone, a culture of the microorganism forms the novel, recoverable, and useful 16α-hydroxy derivative thereof as the first oxidation product.

The microorganism, when grown on agar, has mature vegetative hyphae whose diameter varies from 0.9 to 1.2 microns. The aerial mycelium is hyaline under the microscope, generally branched, not forming loops or spirals. Individual filaments are rarely or not septate. The colour of colonies when viewed on agar without magnification is white to light gull grey (Ridgway plate I. III 10 f). The spores are oval to oblong. Mature spores range from about 1.0 to 1.2 microns in diameter and from 1.0 to 1.2 microns in length. Individual spores are colorless at maturity, but in mass appear white to grey when viewed without magnification (Ridgway plate L III 10 f).

The microorganism will liquefy gelatine, peptonize litmus milk, reduce nitrate to nitrite, and produce hydrogen sulphide when grown on Kligler iron agar. It does not produce indole when grown on tryptone agar. It will grow on

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media containing ammonium sulphate, or sodium nitrate, or asparagine, or tryptophane as sole source of nitrogen (basal medium: KH_2PO_4, 2.38 g.; K_2HPO_4. 5.65 g.; $MgSO_4.7H_2O$, 1.0 g.; $CuSO_4.5H_2O$, 0.0064 g.; $FeSO_4.7H_2O$, 0.0011 g.; $MnSO_4.4H_2O$, 0.0079 g.; $ZnSO_4.7H_2O$, 0.0015 g.; "Difco" agar ("Difco" being a Registered Trade Mark), 15 g.; glucose. 10 g.; water to 1 litre; pH adjusted to 6.8). When the same basal medium without carbohydrate but with 0.106 g. N (as $(NH_4)_2SO_4$) is used, no growth is obtained. Addition of the following sugars (at 10 g./l) supports growth; arabinose, rhamnose, glucose, galactose, fructose, mannose, lactose, maltose, dextrin, starch, glycerol, mannitol, salicin. No growth is noted when sucrose, raffinose, sorbose, sorbitol or inositol are added to the basal medium. Sodium acetate and sodium citrate support growth when added to the basal medium, but sodium tartrate does not.

A pure culture of this microorganism has been filed with the American Type Culture Collection, Washington, D.C., under No. 11,009, and the microorganism is accordingly hereinafter referred to as "actinomycete species ATCC 11,009."

In general, the conditions of culturing actinomycete species ATCC 11,009 for the purposes of this invention are (except for the inclusion of the steroid to be converted) the same as those of culturing various other actinomycetes for the production of antibiotics and/or vitamin B_{12}, i.e. the microorganism is aerobically grown in contact with (in or on) a suitable fermentation medium. A suitable medium essentially comprises a source of nitrogenous and growth-promoting factors, and an assimilable source of carbon and energy. The latter may be a carbohydrate (such as glucose, maltose, starch or dextrin) and/or the steroid itself. Preferably, however, the medium includes an assimilable source of carbon and energy in addition to the steroid: and preferably also, this source is at least in substantial part a member of the group consisting of (1) fat acids having at least 14 carbon atoms and (2) fats. Use of such lipid source of carbon and energy (especially use of a fatty oil) is advantageous in that it enhances the availability of the steroid for conversion. The medium may also include precursors for by-products particularly desired, e.g. an assimilable source of cobalt, where vitamin B_{12} is a desired by-product.

Among the fats utilizable for the purposes of this invention are:—lard oil, soybean oil, linseed oil, cottonseed oil, peanut oil, coconut oil, corn oil, castor oil, sesame oil, crude palm oil, fancy mutton tallow, sperm oil, olive oil, tristearin, tripalmitin, triolein and trilaurin. Among the fat acids utilizable for the purposes of this invention are: stearic acid, palmitic acid, oleic acid, linoleic acid and myristic acid.

The source of nitrogenous and growth promoting factors may be organic (e.g. soybean meal, corn steep liquor, meat extract and/or distillers solubles) or synthetic (i.e., composed of simple synthesizable organic and inorganic compounds).

Among the steroids which may be converted by the practice of this invention are: progesterone; pregnenolone; pregnanolone; 17-hydroxy-11-desoxy-corticosterone (Rechstein's compound S); compound S acetate; desoxycorticosterone; ergosterol; stigmasterol; cholesterol; diosgenin; and desoxycorticosterone acetate. Where an oxidizable hydroxy group is present in the steroid and its oxidation is not desired, it may be converted into a group resistant to oxidation and capable of reconversion into a hydroxyl group; e.g., an ester, ether or halogen group.

The following examples are illustrative of the invention:—

EXAMPLE 1.

An aqueous medium of the following composition is prepared:—

soybean oil	8.8 ml.
progesterone	0.25 g.
soybean meal	30 g.
$CO(NO_3)_2.6H_2O$	0.005 g.
water	1 litre

100 ml. portions of the medium are distributed in 500 ml. Erlenmeyer flasks, and the flasks are plugged with cotton and sterilized in the usual manner (by autoclaving). When cool, each of the flasks is inoculated with 2% of a vegetative inoculum of actinomycete species ATCC 11,009 which has been grown for 48—72 hours on a soybean meal-glucose medium, and the flasks are maintained at 25° C. and mechanically shaken. After two days incubation, the medium has a pH of about 6.3, a vitamin B_{12} content of about 0.15 micrograms/ml., a progesterone content of about 106 micrograms/ml. and a 16α-hydroxyprogesterone content of about 115 micrograms/ml. The presence and quantity of 16α-hydroxyprogesterone and unoxidized progesterone is determined by extracting the sample with chloroform, separating the oxidized compound from progesterone using the filter paper partition chromatographic method of Zaffaroni (Science 111: 6. 1950; propylene glycol-toluene system) and determining the quantity of steroid in

each "spot" using a quartz spectrophotometer (240 mμ). The oxidised steroid appears to predominate in the cell-free filtrate, while the unoxidised steroid is associated with the cells of the micro-organism.

The 16α-hydroxyprogesterone is recovered from the culture filtrate as described hereinafter.

EXAMPLE 2.

(a) An aqueous medium of the following composition is prepared, and distributed, sterilized, inoculated, and incubated as described in Example 1:—

Soybean oil	2.2 ml.
progesterone	0.25 g.
Glycine	2.6 g.
sodium acid glutamate	2.2 g.
$FeSO_4.7H_2O$	0.25 g.
$ZnSO_4.7H_2O$	0.03 g.
$CuSO_4.5H_2O$	0.012 g.
$Co(NO_3)_2.6H_2O$	0.005 g.
$MnSO_4.4H_2O$	0.016 g.
$CaCl_2.2H_2O$	0.05 g.
$MgSO_4.7H_2O$	0.5 g.
$K_2HPO_4.3H_2O$	0.5 g.
water	1 litre

(b) After three days incubation, the (unfiltered) medium from 700 flasks is pooled, and centrifuged; and the clear liquid is extracted with thirteen 4-litre portions of chloroform. The chloroform extract is evaporated to dryness *in vacuo*, and the residue (about 31 g.) is mixed with 400 ml. of 80% methanol. The resulting suspension is extracted with seven 400 ml. portions of hexane (which removes all the lipids and leaves the essentially pure oxidised steroids in the aqueous methanol phase). The aqueous methanol phase is evaporated, leaving about 8.7 g. of a semicrystalline mixture; and the latter is dissolved in 50 ml. chloroform and 50 ml. benzene, and chromatographed on a column (7 cm. diameter) containing 800 g. of a mixture of equal volumes magnesium silicate and a filter aid (such as that known by the Registered Trade Mark "Celite"); and the column (X) is then washed with a mixture of equal volumes of chloroform and benzene. At first, residual progesterone was eluted, followed by a small fraction of crystalline material (Y), and then by the main product of the fermentation, 16α-hydroxyprogesterone, which is recovered from the eluate by evaporation of the solvents and then crystallised from acetone.

The 16α-hydroxyprogesterone forms hexagonal crystals melting at 225°—226° C. It gives a blue coloration with iodine and KI solution on filter paper.

$[\alpha]_D^{23}+170°$ (C, 0.38 in chloroform).

U.V.: $\lambda_{max.}^{alc.}$ 239 mμ(ε=17,000).

I.R.: $\lambda_{max.}^{Nujol}$ 3.04 (hydroxyl); 5.90 (20-keto); 6.05 and 6.20 (3-keto, Δ4.5). Its analysis (C, 76.61; H, 9.56) is in good agreement with that calculated for $C_{21}H_{30}O_3$. ("Nujol" is a Registered Trade Mark).

Acetylation of 16α-hydroxyprogesterone with acetic anhydride and pyridine yields a monoacetate, which on crystallization from acetone and hexane melts at 134°—135° C., has $[\alpha]_D^{22}+107°$, and gives the following figures on analysis: C, 73.64; H, 8.61; and acetyl, 11.63. Esters of 16α-hydroxyprogesterone with other organic acids (e.g., benzoic acid) may be obtained analogously.

The crystalline material Y obtained from the second eluate by evaporation of the solvent and repeated crystallization from acetone melts at 199°—200° C., and has the characteristics following:—

$[\alpha]_D^{23}+90.5°$ (C, 0.82 in chloroform).

U.V.: $\lambda_{max.}^{alc.}$ 284 mμ(ε=65).

Analysis: C, 76.12; H, 9.73 (in good agreement with that calculated for $C_{21}H_{32}O_3$). The product is a 4,5-dihydro-16α-hydroxyprogesterone. It may be converted into its acetate (or other ester) in the same manner as 16α-hydroxyprogesterone.

Further elution of the silicate-filter aid column X with pure chloroform yields an additional quantity of 16-hydroxyprogesterone. The column is then further eluated with a mixture of three parts chloroform and one part acetone; and the solvent is removed from this eluate by evaporation and the residue crystallized from acetone. The product melts at 215.5°—216.5° C., and has the following characteristics: $[\alpha]_D^{24}-39°$ (chloroform): U.V. $\lambda_{max.}^{alc.}$ 243 mμ (ε=14,400). Its analysis (C, 73.09; H, 8.68 is in good agreement with that calculated for $C_{21}H_{30}O_4$, the product being believed to be a dihydroxyprogesterone with one of the hydroxy groups in 16-position

EXAMPLE 3.

Duplicate of Example 1 using a medium of the following composition:—

soybean oil	6.8 ml.
progesterone	0.25 g.
dried brewers yeast	25.0 g.
$Co(NO_3)2.6H_2O$	0.005 g.
water	1 litre

After two days incubation, the medium has a pH of about 6.3 a vitamin B_{12} content of about 0.20 micrograms/ml., and a 16α-hydroxyprogesterone content of about 130 micrograms/ml. (about 120 micrograms/ml. in the culture filtrate).

EXAMPLE 4.

Duplicate of Example 1 using a medium of the following composition:—

soybean oil	8.8 ml.
progesterone	0.25 g.
$Co(NO_3)_2.6H_2O$	0.005 g.
cornsteep liquor (neutralized to pH 7)	40 g.
$CaCO_3$	5 g.
water	1 litre

After three days incubation, the medium has a pH of about 6.5, a vitamin B_{12} content of about 0.21 micrograms/ml., and a 16α-hydroxy-progesterone content of about 95 micrograms/ml. (about 85 micrograms/ml. in the culture filtrate).

On incubating for four days, both the vitamin B_{12} and 16α-hydroxyprogesterone titers of the medium are materially increased. Thus, on extension of the incubation in Example 3, to four days, the vitamin B_{12} content is raised to 0.43 micrograms/ml. and the 16α-hydroxy-progesterone content to 150 micrograms/ml. (140 micrograms/ml. in the culture filtrate).

In some cases, a further increase in titer may be obtained on further extension of the incubation period. Thus, on extension of the incubation in Example 4, to 7 days, the 16α-hydroxyprogesterone content is raised to 180 micrograms/ml. (175 micrograms/ml. in the culture filtrate).

In the Examples given hereinbefore, the steroid precursor is progesterone; but other steroid precursors can be converted into useful steroid derivatives, as illustrated by the following examples:—

EXAMPLE 5.

(a) 3 g. compound S acetate is incorporated in 15 litres of a soybean oil medium of the following composition:—

glycine	2.6 g.
sodium acid glutamate	2.1 g.
$MgSO_4.7H_2O$	0.5 g.
$K_2HPO_4.3H_2O$	0.5 g.
$ZnSO_4.7H_2O$	0.03 g.
$MnSO_4.4H_2O$	0.016 g.
$CuSO_4.5H_2O$	0.012 g.
$FeSO_4.7H_2O$	0.025 g.
$CaCl_2.H_2O$	0.05 g.
soybean oil	2.2 g.
water	1 litre

The medium is then inoculated with actinomycete species ATCC 11,009 and incubated in a shake flask as described hereinbefore for 4 days. The unfiltered medium is extracted with three 15-litre portions of chloroform, and the solvent evaporated from the extract; and the residue is taken up in 4 litres benzene, and the benzene solution (W) is extracted eight times with 8-litre portions of water. The aqueous phase (Z) is then extracted with 6.4 litres benzene (extract W^1); and the benzene extracts W and W^1 are combined, the benzene removed by evaporation, and the residue recrystallized from alcohol, yielding about 1.1 g. of recovered crude compound S acetate.

(b) The aqueous phase Z is extracted with chloroform, the chloroform is evaporated from the extract and the residue is taken up in hot 95% ethanol. On standing, a precipitate is formed, the product melting at about 208°—213° C.; and an additional yield of the product is obtained by evaporating the mother liquor to half-volume. Repeated recrystallisation from ethanol yields the product as characteristic diamond-shaped platelets melting at about 224°—227° C. Acetylation of the product in pyridine-acetic anhydride yields needles melting at 209°—211° C. $[\alpha]_D^{25} -58°$ (0.53% in chloroform). Analysis: C, 67.5; H, 8.08. The product appears to be a hydroxy derivative of compound S.

EXAMPLE 6.

1 g. 11-desoxycorticosterone acetate is incorporated in 5 litres of the soybean oil medium described in Example 5 and the medium is inoculated and incubated as described in that Example for 5 days. The unfiltered medium is extracted with three 5-litre portions of chloroform, the solvent evaporated from the extract, and the residue partitioned between benzene and water. On further purification, the aqueous phase yields an oxidized derivative of desoxycorticosterone, as indicated by mobility in filter-paper partition chromatography using Zaffaroni's methods.

EXAMPLE 7.

100 mg. pregnenolone is incorporated in 500 ml. of the soybean oil medium described in Example 5, and the medium is inoculated and incubated as described in that example for 3 days. The unfiltered medium is extracted once with 500 ml. chloroform and again with 250 ml. chloroform, and the extract further treated to yield a mixture of oxidized derivatives of pregnenolone, probably 16α-hydroxyprogesterone and progesterone (based on filter-paper partition chromatography).

Other media than those disclosed in the foregoing examples may be used for the purpose of this invention, the only requirement being of course that they be media which support the aerobic growth of actinomycetes. The vitamin B_{12} content of the media may be recovered by the conventional procedures for recovery from actinomycete cultures. An adequate (sterile) air supply should be maintained during the fermentation; which may be

done in the conventional manner of conducting aerobic fermentations, e.g. by exposure of a large surface of the medium to air or by submerged aerated culture. The incubation time may determine the degree of oxidation. Thus, pregnenolone appears to be oxidized first to progesterone and on further incubation the latter is converted to 16α-hydroxyprogesterone. The incubation may of course be stopped at a time when the medium contains the highest titer of progesterone, if that is the desired product.

In the Examples given hereinbefore, the steroid is included in the fermentation medium before inoculation with the microorganism; but the steroid may be added after the inoculation and even after substantial growth of the microorganism has occurred, as illustrated by the example following. Where an intermediate stage of oxidation is desired, as for example progesterone rather than 16α-hydroxyprogesterone, it is favoured by a shorter fermentation period.

EXAMPLE 8.

An aqueous medium of the following composition is prepared, and distributed, sterilized, inoculated, and incubated as described in Example 1.

soybean meal	15 g.
glucose	10 g.
soybean oil	2 g.
water	1 litre

After incubating for one day, pregnenolone is added to each flask in the proportion of 200 mg./litre medium; and the medium is further incubated for six hours, and the pooled medium then extracted with three 1-litre portions of chloroform. The pooled chloroform extract contains about 190 mg. progesterone.

In the Examples given hereinbefore, the steroid is converted by inclusion in an aerobic culture of the microorganism; but the conversion can also be effected by bringing together the steroid and air in an aqueous suspension of the non-propagating microorganism (or by bringing together the steroid, air and enzymes of the microorganism in an aqueous cell-free medium), as illustrated by the following example:—

EXAMPLE 9.

The two-day culture of actinomycete species ATCC 11,009 described in Example 1 is centrifuged, resuspended in distilled water, recentrifuged, and again resuspended in distilled water. 40 ml. aliquots of the suspension are placed in 125 ml. Erlenmeyer flasks, 10 mg./litre of a polyoxyethylene ether of a partial higher fatty acid ester of sorbitan (such as that known by the Registered Trade Mark "Tween") and 200 mg./litre of pregnenolone are added to each flask, and the flasks are agitated (on a reciprocating shaker) at 25° C. for 24 hours. The pregnenolone added is almost quantitatively converted to progesterone, which may be recovered by chloroform extraction.

16α-hydroxyprogesterone may be converted into the known 17-hydroxyprogesterone by dehydrating the former with aluminium tertiary butylate to obtain 16-dehydroprogesterone, and converting the dehydro compound into the 17-hydroxy compound by the method described by Julian et al. in J.A.C.S. 72, 367 (1950). The dehydration may, for example, be effected as follows: A solution of 300 mg. aluminium tertiary butylate in 10 ml. benzene is added to a solution of 97 mg. 16α-hydroxyprogesterone in 5 ml. dry acetone, the mixture is refluxed for 12 hours and the resulting gel is decomposed by the addition of dilute sulphuric acid. The benzene layer formed is separated from the aqueous layer, and the latter extracted with chloroform; and the combined benzene layer and chloroform extract is washed with dilute sodium bicarbonate solution and water, dried over sodium sulphate, and evaporated to dryness in vacuo.

The residue (about 91 mg.) is dissolved in a mixture of 3 ml. benzene and 3 ml. hexane, and chromatographed on 4 g. sulphuric acid-washed alumina. Elution with a 2:1 benzene-hexane mixture yields pure 16α-dehydroprogesterone as thin platelets, which after recrystallization from an acetone-hexane mixture melts at 190°—191.5° C., and has the following properties:—

$[\alpha]_D^{23}$ +134.5° (concentration, 0.90 in chloroform); U.V.: $\lambda_{max.}^{alc.}$ 240 mμ (ε= 28,400), 315 mμ (ε=148).

These properties are in good agreement with those reported for 16α-dehydroprogesterone by Fukushima and Gallagher J.A.C.S. 73, 201 (1951); and the analysis (C, 80.94%; H, 8.99%) is in good agreement with that calculated for $C_{21}H_{28}O_2$.

What we claim is:—

1. A hydroxy-progesterone having the or one of the hydroxy groups in the 16α position, which is formed by subjecting progesterone to the action of the enzymes of actinomycete species ATCC 11,009 under aerobic conditions.

2. 16α-hydroxyprogesterone.

3. An ester of 16α-hydroxyprogesterone.

4. 4.5 - dihydro - 16α - hydroxyprogesterone.

5. An ester of 4,5-dihydro-16α-hydroxyprogesterone.

6. A dihydroxy-progesterone formed by subjecting progesterone to the action of enzymes of actinomycete species ATCC 11,009 under aerobic conditions.

7. The method of converting a 3-keto steroid into a hydroxylated derivative thereof which comprises subjecting the steroid to the action of enzymes of actinomycete species ATCC 11,009 under aerobic conditions.

8. The method of converting progesterone into a hydroxylated derivative thereof having the or one of the hydroxy groups in the 16α position which comprises subjecting the progesterone to the action of enzymes of actinomycete species ATCC 11,009 under aerobic conditions.

9. The method as claimed in Claim 7, wherein the action is effected in a medium which contains an assimilable source of cobalt.

10. The method of converting a 3-keto steroid into a hydroxylated derivative thereof, which comprises including the steroid in an aerobic culture of actinomycete species ATCC 11,009, and recovering the hydroxylated derivative from the culture.

11. The method of converting a 3-keto steroid into a hydroxylated derivative thereof, which comprises bringing together, in an aqueous medium, the steroid, air, and enzymes of non-propagating cells of actinomycete species ATCC 11,009.

12. The method of converting a 3-keto steroid into a hydroxylated derivative thereof, which comprises incorporating the steroid in a fermentation medium capable of supporting the growth of an actinomycete, inoculating the medium with actinomycete species ATCC 11,009, incubating the medium under aerobic conditions, and recovering the hydroxylated derivative from the incubated medium.

13. The method as claimed in Claim 12, wherein the fermentation medium comprises, as at least a substantial part of the requisite assimilable source of carbon and energy, a member of the group consisting of (1) fat acids having at least 14 carbon atoms and (2) fats.

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